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ORIGINAL ARTICLE

The effect of chronic immobilization stress on leptin signaling in the ovariectomized (OVX) rat

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Abstract Previous studies have shown that both 17β-estradiol (E2) treatment and chronic stress may attenuate post-OVX weight gain in the female rat. However, the interaction between E2 and stress is unclear. This study examined the effect of E2 treatment and chronic immobilization stress on body weight. Adult OVX Sprague–Dawley rats were randomly assigned to one of four treatment groups in a 2X2 factorial design examining hormone treatment [vehicle (VEH) or E2, sc] and stress (no stress vs stress 60 min/day for 22 days). After 22 days, E2 significantly inhibited weight gain and food intake in OVX rats. In contrast, chronic stress reduced body weight only in control OVX animals but did not affect food intake. E2 reduced circulating leptin levels in non-stressed animals, but not in animals subjected to chronic immobilization. Western blot analysis indicated that E2 treatment increased leptin receptor (Ob-Rb) expression in the medial basal hypothalamus (MBH); however, this treatment also increased suppressor of cytokine signaling 3 (SOCS3), which is an inhibitor of leptin signaling. Chronic immobilization stress blunted the E2-induced increase in Ob-Rb and SOCS3 levels. These results suggest that chronic stress counteracts E2 effects on leptin signaling in the MBH without altering body weight.

Keywords Stress · Leptin · Immobilization · Body weight · Estrogen receptor

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Introduction

Menopausal women have reduced levels of estrogen, which is associated with an increased risk of developing central obesity, diabetes, and cardiovascular disease [17, 22, 56]. Furthermore, estrogen may directly play a role in the distribution of adipose tissue since estrogen receptor (ER) expression has been measured in human preadipocytes [27]. The OVX rat has been used as a model to study the complex interaction between estrogen and body weight. Removal of the ovaries induces a hypoestrogenic state resulting in excess accumulation of abdominal fat and the release of proinflammatory cytokines from the liver, whereas estrogen replacement reverses these effects [44, 45]. In non-pregnant female rats the predominant estrogen is E2, and its actions are mediated through the activation of two ER isoforms, ERα and ERβ [13, 14, 43]. Our lab and

others have shown that selective activation of ERα attenuates post-ovariectomy weight gain in OVX and OVX/adrenalectomized rats implicating ERα mediated regulation of body weight [48, 53]. Consistent with this, transgenic mice deficient in ERα, but not ERβ, become hyperphagic and obese compared to corresponding wild-type mice [42].

Regulation of feeding and body weight is partly a consequence of circulating leptin, a hormone of adipose origin. Leptin is transported across the blood brain barrier [4] and acts centrally since an intracerebroventricular (ICV) injection of leptin in mice reduces food intake and increases energy expenditure [21]. Similarly, knockout mice deficient in the long form of the leptin receptor (Ob-Rb) develop severe obesity and are unresponsive to leptin treatment thereby implicating this receptor as the signal-transducing element for the effect of leptin on body weight [11]. Ligand-activated Ob-Rb induces the phosphorylation of the signal transducer and activator of transcription 3 (STAT3) [3] to regulate the expression of pro-opiomelanocortin (POMC) and SOCS3, in turn, modulating energy homeostasis [29, 39, 50].

Interactions between circulating E2 and leptin to regulate feeding and body weight have been reported and the hypothalamic arcuate nucleus (ARC) is a central integrative loci for both effects [9]. E2 treatment has been shown to increase leptin synthesis and secretion in adipose tissue [31]; and up-regulating Ob-Rb levels in the hypothalamus and white adipose tissue [36]. However, other studies indicated that treatment with E2 in leptin and leptin receptor deficient mice significantly reduced food intake and body weight suggesting that E2 can elicit its effects regardless of leptin status [20]. These investigators further reported that E2 activated the STAT3 pathway, thereby mimicking leptin signaling, and that brain-specific STAT3 knockout mice were unresponsive to E2 administration suggesting E2's mode of action is STAT3-dependent [19]. Consequently, E2 seems to act downstream of Ob-Rb to activate STAT3 and facilitate transcription of target genes including POMC to regulate food intake [19, 20].

In animal models the effects of stress on body weight depend largely on the type of stressor applied [1]. For the most part, physical stressors such as immobilization stress reduce food intake and body weight in male rats [1, 33]. However, the interaction between estrogens and stress in the control of body weight has not been carefully explored, particularly because most studies have focused on males. Male rats are reportedly more sensitive to stress-induced changes in feeding and body weight than females, suggesting that gonadal hormones may play an important role in mediating stress effects on metabolism [15]. Furthermore, insight from another chronic stress model where male rats were treated with dexamethasone (DEX), a synthetic glucocorticoid, resulted in elevated plasma leptin, reduced feeding, and lower body weight compared to untreated controls [25]. Such results indicate a potential interaction between the stress response and leptin; yet, whether this is the case for the female rat is still not clear.

The experiments presented here examined the effects of chronic stress and E2 treatment on leptin signaling in post-OVX body weight regulation. Hormone-treated OVX Sprague–Dawley rats were immobilized daily to induce a chronic stress state. The effects of hormone treatment and chronic stress on leptin levels were determined in addition to corticosterone (CORT) levels as a measure of hypothalamic–pituitary–adrenal (HPA) activity. Finally, the relative levels of downstream effectors of leptin signaling were analyzed across groups within the medial basal hypothalamus (MBH) including Ob-Rb, phosphorylated STAT3 at tyrosine residue 705 (p-STAT3), and SOCS3.

Materials and methods

Subjects

Forty-eight female Sprague–Dawley rats (Harlan, Indianapolis, IN; 200–225 g) were pair-housed in standard polycarbonate shoebox cages (42 × 20.5 × 20 cm) containing hardwood chip bedding (Sani-Chip, Harlan Teklad, Madison, WI). All animals had ad libitum access to soy-free rodent chow (Dyets, Inc. Bethlehem, PA) [54] and water. The housing room was maintained at 22–25 °C at 50 % humidity on a 12-h reverse light/dark cycle. Upon arrival, all animals were individually handled daily and acclimated to facilities and equipment before commencement of experiments.

Body weight, food intake, and water consumption were measured daily over the entire length of the study—subjects were individually weighed, while food and water consumption were recorded for each pair. All procedures conducted were approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences and conducted in full compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Experimental design

The experiment was conducted as a 2 (no stress or 60 min immobilization/day) × 2 (VEH or E2) factorial design with 12 animals per treatment group for a total of 48 animals. Groups were counter-balanced for body weight before any experimental manipulation.

Drugs

17β-Estradiol (E2) (Sigma Chemical Co., St Louis, MO), binds with nearly equal affinity to both ERs [30].

OVX and osmotic pump implant

Nine days after arrival, all animals underwent bilateral ovariectomy and osmotic mini-pumps (Alzet Model 2002 0.5ML, Durect Co., Cupertino, CA, USA) were implanted subcutaneously between the shoulder blades while the animal was under isoflurane anesthesia. Pumps contained either VEH or E2. E2 was dissolved in VEH (27 % hydroxypropyl-β-cyclodextrin in sterile water, Acros Organics, Fair Lawn, NJ) [54]. On post-implant day 13, pumps were replaced with a new pump while the animal was under isoflurane anesthesia. Mini-osmotic pumps release their contents at a slow continuous infusion rate of 0.5 µl/h and delivered 0.25 mg/kg body weight (bw)/day of E2. Control animals in both the stress and non-stress groups received VEH only.

Immobilization stress

Four days after ovariectomy and mini-pump implantation rats assigned to the stress groups were immobilized for 60 min/day for 22 days using finger-like restraining devices (Centrap Cages, Fisher Scientific). Immobilization stress was initiated 1 h after lights out, during the active phase of the day [47]. Rats were placed in the Centrap cage and the restraining "fingers" were tightened until the animals were immobilized but not compressed, pinched, or in pain. This device allows for very little movement. Animals were unable to turn or barrel-roll in the Centrap cage. This restraint procedure, acute or chronic, reliably produces elevations in hormones associated with a stress response including ACTH and CORT [51].

Collection of serum and brain tissue

All rats were killed by carbon dioxide overdose with rapid decapitation within 1 h after the end of the final immobilization. Brains were quickly removed, snap-frozen on dry ice, and stored at −80 °C. Trunk blood was collected into ice-cold tubes followed by centrifugation at 4,000×*g* for 20 min. Blood serum was extracted and stored at −80 °C before further analysis.

Western blot

The MBH was dissected on ice as previously described [23, 49]. Brain tissue was either homogenized in RIPA buffer (50 mM Tris–HCl pH 7.5; 150 mM NaCl; 1 % NP-40; 1 % NaDOC; 0.1 % SDS; Roche Complete Protease Inhibitor, Indianapolis, IN) for measuring Ob-Rb expression or O'dell's buffer (50 mM Tris–HCl pH 7.5; 50 mM NaCl; 10 mM EGTA; 10 mM EDTA; 80 µM Na_2MoO_4; 5 mM $NaPO_4$; 1 mM Na_3VO_4; 1 mM PMSF; 4 mM pNPP; 1 % Triton; Sigma Cocktail I and II; Roche Complete Protease Inhibitor, Indianapolis, IN) for phosphorylated proteins. Lysates were centrifuged at 20,000×g at 4 °C for 30 min and subjected to SDS-PAGE under reducing conditions using 4–15 % Tris–HCl gels (Bio-Rad, Hercules, CA) according to a previously established method [12]. The resolved proteins were electroblotted onto a polyvinyldifluoride membrane (Bio-Rad, Hercules, CA) in a Tris/glycine buffer (25 mM Tris, 0.192 M glycine, 0.01 % SDS, pH 8.3) and blocked overnight with 5 % (w/v) non-fat dry milk in Tris buffered saline (TBS: 25 mM Tris–HCl, 125 mM NaCl, pH 7.4) before probing overnight at 4 °C with a specific antibody (anti-mouse monoclonal Ob-Rb' 1:500, Santa Cruz Technologies, Santa Cruz, CA; monoclonal anti-rabbit p-STAT3 (Y705) 1:1000, STAT3 1:1000; Cell Signaling, Danvers, MA). Blots were subsequently washed three times with 0.1 % Tween-TBS and subjected to 1 h incubation at room temperature with the appropriate secondary antibody conjugated to a peroxidase enzyme. The blots were visualized with a chemiluminescent signal (SuperSignal West Femto chemiluminescence kit; Pierce Chemical Co., Rockford, IL) subsequently digitized (Fujifilm LAS-3000 imager; Stamford, CT) and analyzed (Fujifilm Image Gaug; Valhalla, NY). Loading amounts were determined by Coomassie blue staining (CBS) (Bio-Rad, Hercules, CA). All values are expressed relative to non-stressed VEH-treated animals.

Enzyme-linked immunosorbent assays (ELISA)

A competitive ELISA kit was used to determine the serum concentrations of CORT and E2 (Cayman Chemical, Ann Arbor, MI); intra-assay coefficients of variance were 5 and 6 %, respectively. A sandwich ELISA kit was used to determine leptin levels (SPI Bio, Montigny Le Bretonneux, France); intra-assay coefficients of variance were 7 %.

Statistical analysis

Using the statistical software SPSS 16.0 (Chicago, IL), hormone and stress interactions were examined using two factor analysis of variance followed by Fischer's least significant difference post hoc test. Leptin and CORT values were log transformed to insure equal variance between groups. Body weight and cumulative food intake were analyzed by repeated measures analysis. A value of $P < 0.05$ was considered significant.

Results

Body weight and food consumption

We examined the effect of E2 and chronic immobilization stress on body weight gain. Repeated measures analysis

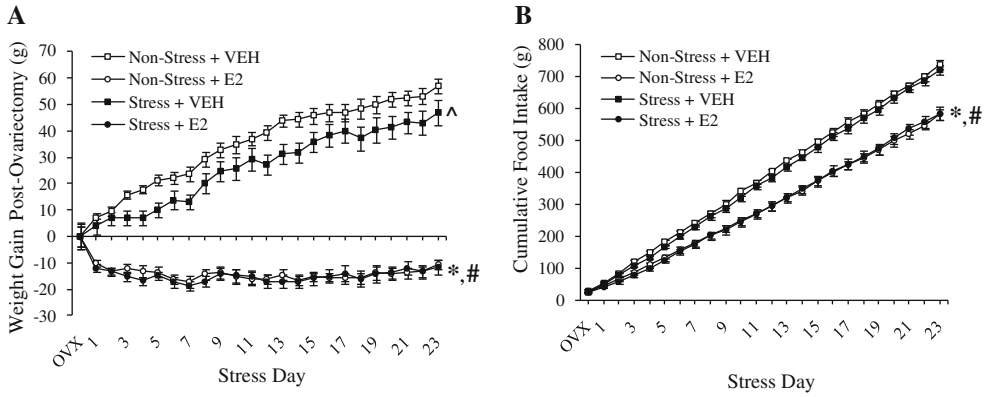

Fig. 1 Effect of hormone treatment and stress on body weight and daily cumulative intake. **a** E2 treatment prevented post-ovariectomy weight gain in female rats compared to VEH treatment. Stressed VEH-treated rats had decreased weight gain relative to non-stressed controls. **b** Rats treated with E2 had decreased cumulative food intake compared to rats treated with VEH only. Stress had no effect on food intake. Data points represent daily mean body weight values ± SEM of $n = 12$ animals per group. *Non-stressed E2-treated rats versus non-stressed VEH-treated rats ($P < 0.05$). ^Stressed VEH-treated rats versus non-stressed VEH-treated rats ($P < 0.05$). #Stressed E2-treated rats versus stressed VEH-treated rats ($P < 0.05$)

revealed a significant effect of hormone [$F(1,28) = 466.268$, $P < 0.05$], stress [$F(1,28) = 7.248$, $P < 0.05$], and an interaction [$F(1,28) = 6.313$, $P < 0.05$]. Both non-stress and stress OVX rats treated with E2 for 22 days had decreased body weight ($P < 0.05$) compared to rats treated with VEH (Fig. 1a). Furthermore, VEH-treated rats exposed to chronic stress had significantly ($P < 0.05$) decreased weight gain compared to non-stressed controls. There were no significant differences in weight gain between non-stress and stress E2-treated rats (Fig. 1a).

To determine whether hormone treatment or chronic stress effects on body weight were due to changes in food consumption, we measured daily food intake from all groups. Daily values were summed and the cumulative food intake was calculated. Analysis of food intake revealed an effect of hormone treatment [$F(1,20) = 67.884$, $P < 0.05$], but not of stress [$F(1,20) = 0.370$, $P > 0.10$] or a hormone treatment × stress interaction [$F(1,20) = 0.383$, $P > 0.10$]. E2 treatment significantly ($P < 0.05$) reduced daily food intake independent of stress exposure (Fig. 1b).

Serum analysis

Since our results demonstrated an effect of hormone and stress on weight gain, we assessed the effect of chronic stress and E2 treatment on markers of metabolism and HPA activity. We measured serum leptin and CORT on the final day of the stress phase in all animals. Stressed rats were immobilized for 60 min and blood was collected within 1 h following the cessation of immobilization. Serum levels of E2 were also measured to confirm E2-treated animals had higher levels of E2 than VEH-treated animals; and to determine whether there was any effect of stress on E2 levels.

Analysis of leptin levels revealed a significant hormone treatment effect [$F(1,44) = 5.88$, $P < 0.05$], an effect of stress [$F(1,44) = 9.231$, $P < 0.05$] and an interaction [$F(1,44) = 11.364$, $P < 0.05$]. Non-stressed rats treated with E2 had lower serum leptin ($P < 0.05$) compared to rats receiving only VEH. Interestingly, the E2-induced reduction in serum leptin was prevented by chronic immobilization stress (Fig. 2a).

To determine differences in HPA activity, serum CORT levels were determined in all groups. There was a significant effect of hormone treatment [$F(1,44) = 11.931$, $P < 0.05$], stress [$F(1,44) = 26.058$, $P < 0.05$], and an interaction [$F(1,44) = 4.018$, $P < 0.05$]. Chronic stress significantly ($P < 0.05$) decreased CORT levels while E2 treatment inhibited this repression (Fig. 2b).

Analysis of E2 levels revealed a significant effect of hormone treatment [$F(1,28) = 75.571$, $P < 0.05$] but not an effect of stress [$F(1,28) = 1.694$, $P > 0.05$] or an interaction [$F(1,28) = 1.445$, $P > 0.05$]. Non-stressed and stressed rats treated with E2 had significantly ($P < 0.05$) greater levels of circulating E2 relative to VEH-treated rats (Fig. 3). Stress had no effect on E2 levels between groups receiving the same hormone treatment (Fig. 3).

Chronic stress alters E2-induced changes in leptin signaling

We examined the effect of E2 treatment and chronic immobilization stress on certain components of the leptin signaling pathway within the MBH containing mostly the ARC. Analysis of Ob-Rb levels in the MBH did not reveal a significant hormone effect [$F(1,12) = 1.904$, $P > 0.10$]

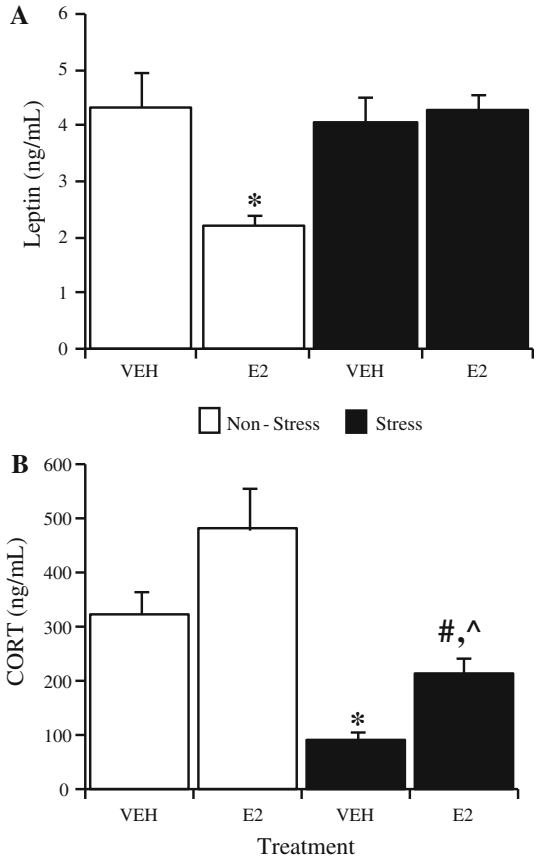

Fig. 2 Effect of hormone and stress on serum leptin and CORT. Blood serum was extracted from all subjects on the final day of stress (Post-OVX day 26) and analyzed for leptin and CORT. **a** Leptin levels in animals treated with E2 were significantly lower than VEH-treated rats while stress blunted this effect. **b** Daily exposure to immobilization for 22 days significantly decreased CORT levels; however, this effect was diminished by E2 treatment. *Bars* represent mean values ± SEM of $n = 12$ animals per group. *Versus non-stressed VEH-treated rats ($P < 0.05$). #Versus stressed VEH-treated rats ($P < 0.05$). ^Versus non-stressed E2-treated rats ($P < 0.05$)

or stress effect [$F(1,12) = 0.897$, $P > 0.10$], however, there was a significant interaction [$F(1,12) = 5.710$, $P > 0.05$]. Post hoc analysis indicated E2 treatment significantly increases ($P < 0.05$) expression in the MBH relative to control animals while chronic stress exposure repressed this effect (Fig. 4a).

We also measured p-STAT3 (Y705) and SOCS3 levels in the MBH. There was no effect of hormone treatment [$F(1,12) = 1.115$, $P > 0.10$], stress [$F(1,12) = 3.004$, $P > 0.10$] or an interaction [$F(1,12) = 0.203$, $P > 0.10$] on p-STAT levels (Fig. 4b). Analysis of SOCS levels revealed hormone treatment [$F(1,12) = 0.582$, $P > 0.1$] or stress [$F(1,12) = 0.285$, $P > 0.1$] alone did not have a significant effect. However, there was a significant interaction of hormone treatment and stress [$F(1,12) = 9.478$, $P < 0.05$]. E2 treatment increased ($P < 0.05$) SOCS3 expression while chronic stress exposure blunted this effect (Fig. 4c).

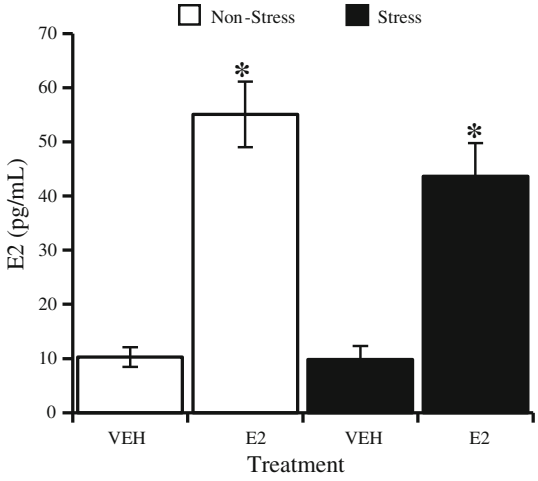

Fig. 3 Effect of hormone and stress on serum E2. As expected, the levels of E2 in E2-treated rats were significantly higher than VEH-treated rats. *Bars* represent mean values ± SEM of $n = 8$ animals per group. *Versus VEH-treated rats ($P < 0.05$)

There was no significant difference in SOCS3 levels between non-stressed and stressed rats treated with VEH.

Discussion

Consistent with previous findings [35, 48, 53], the present study shows that E2 treatment attenuates post-ovariectomy weight gain. These animals also had reduced circulating leptin with an increase in leptin receptor levels in the MBH. Chronic immobilization stress blocked the ability of E2 to reduce serum leptin and increase Ob-Rb levels in the MBH. However, chronically stressed animals treated with E2 were indistinguishable from non-stressed E2-treated rats in the amount of weight lost and food consumed post-ovariectomy. Interestingly, VEH-treated stressed rats gained less weight than non-stressed VEH-treated rats without any differences in cumulative food intake. This effect seemed to be independent of the low levels of E2 present after removal of the ovaries since there was no difference in E2 levels between non-stressed and stressed VEH-treated rats.

E2 regulation of leptin signaling by increasing the levels of hypothalamic Ob-Rb have been previously reported indicating an E2 and leptin interaction to regulate body weight [20, 36]. This interaction is made clearer in a study that found OVX rats treated centrally with leptin were resistant to its anorectic effect suggesting E2 deficiency impairs the effect of leptin to inhibit food intake [2]. To further investigate the role of E2 on leptin signaling in the MBH, we examined downstream effectors of Ob-Rb activation such as STAT3 and SOCS3 [3, 20, 29, 39, 50]. Interestingly, we did not observe an E2-induced change in

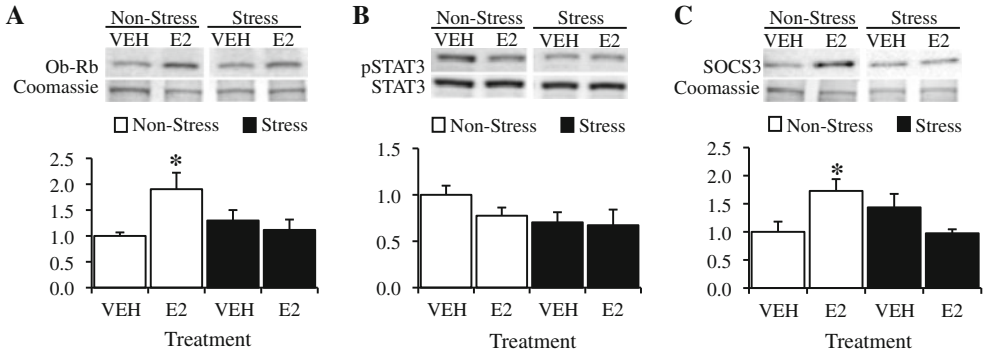

Fig. 4 Effect of hormone treatment and stress on leptin signaling in the MBH. Relative levels of Ob-Rb, pSTAT3, and SOCS3 were measured in the MBH by western blot analysis. **a** E2 treatment significantly increased ($P < 0.05$) Ob-Rb expression in the MBH relative to control animals, an effect that was not observed in chronically stressed E2-treated rats. **b** Hormone or stress had no significant effect on pSTAT (Y705) levels. **c** E2 treatment increased SOC3 levels. Chronic stress exposure inhibited the E2-induced increases in SOCS3. *Bars* represent mean values ± SEM of $n = 4$ animals per group (*CBS* coomassie blue stain). *Versus non-stressed VEH-treated rats ($P < 0.05$)

p-STAT3 levels, which is necessary for dimerization and subsequent translocation to the nucleus for transcription of target genes. This discrepancy may in part be explained by the method we chose to deliver E2 via an osmotic pump. Using this protocol OVX rats in the E2-treated group received a continuous flow of E2. Any E2-induced change in p-STAT3 levels would have likely been transient since a previous report showed hypothalamic p-STAT3 peaked after 60 min of an intraperitoneal injection of E2, subsequently returning to basal levels [20]. Furthermore, it is possible that chronic E2 treatment eventually activates a negative feedback mechanism to modulate STAT3 responsiveness. The mRNA levels of SOCS3, which is a known repressor of leptin and STAT3 signaling [38], have been shown to be under E2 regulation [52]. Indeed, in our study we also show chronic E2 treatment increases SOCS3 levels, which may serve to prevent hyperactivation of STAT3 in conditions where E2 levels remain constant. Whether E2 modulation of SOCS3 is leptin-dependent is not clear in our study. However, previous work suggests both leptin and E2 signal via STAT3 to regulate the levels of POMC and SOCS3 and, subsequently, energy balance [20, 29, 50]. Future studies should consider how these effectors fluctuate in expression levels throughout the day using our experimental conditions.

Our results show that daily immobilization stress for 22 days decreased body weight in VEH-treated rats but not in animals treated with E2. This effect seemed to be independent of locomotor activity (data not shown) and food consumption. Previous studies have shown body weight decreases with repeated exposure to restraint stress; however, most of these studies have limited their investigation to male rodents [10, 40]. Interestingly, we did not observe a stress-induced decrease in the body weight of E2-treated rats, suggesting long-term E2 treatment may be protective against this phenotype. These findings contrast previous work where investigators reported a stressed-induced decrease in the weights of E2-replaced OVX rats [8]. This discrepancy likely reflects the concentration of E2 administered and the protocol used to induce a chronic stress state. In our immobilization protocol unlike restraint stress, animals are unable to barrel roll and thus remain immobilized for the duration of the stress period. Furthermore, the previous study restrained their animals for 6 h daily therefore potentially exacerbating the effect of stress on body weight. It is possible that increasing the duration of stress exposure using our immobilization protocol may result in a stress-induced decrease in body weight of E2-treated rats; however, this remains to be determined. While the stress-induced decrease in the body weights of OVX rats treated with VEH may in part be explained by the up-regulation of stress-sensitive effector molecules mobilized within the hypothalamus and hippocampus as a result of sustained activation of the HPA axis. Some of these effectors such as brain-derived neurotrophic factor (BDNF) have been shown regulate body weight composition and energy expenditure [28, 40, 41]. However, it remains to be determined whether our chronic immobilization paradigm alters the levels of BDNF or other regulators of body weight also associated with the stress response.

Acute activation of the HPA axis leads to the secretion of glucocorticoids followed by a reduction in feeding. Short-term exposure to the synthetic glucocorticoid DEX, increases leptin, reduces feeding and body weight in male rats [25]. This observation suggests an acute interaction between glucocorticoids and leptin to reduce food intake. Consistent with these findings, we show chronically stressed rats treated with E2 had elevated leptin levels; however, these animals did not differ in body weight relative to

non-stressed E2-treated rats at any point throughout the study. Such a response suggests that E2-treated animals may have reached a point where further loss of fat and weight would have been deleterious. On the other hand, leptin levels in stressed VEH-treated rats did not increase relative to non-stressed VEH-treated animals even though there was stressed-induced decrease in body weight. This could be due to increased adiposity as a result of ovariectomy and E2 deficiency leading to elevated leptin levels in both non-stressed and stressed groups as circulating leptin has been shown to correlate with body fat distribution [18, 32]. Any stress-induced differences in leptin would have likely diminished due to increasing fat stores while this was not the case for E2 treated animals.

To assess the HPA axis response to chronic immobilization stress, we measured CORT levels across all groups 1 h after the end of the immobilization stressor. Samples were taken shortly after lights out thus levels reflect the elevation in CORT that normally occurs with the active period. Our results show chronically stressed rats regardless of hormone treatment had diminished CORT. This finding is rather interesting since a previous study using the same immobilization protocol reported females exposed to daily immobilization for 13 days had increased ACTH and CORT levels, which were measured around the same time of the dark cycle we used in this study [16]. However, with the current results, it is difficult to interpret whether chronic immobilization stress actually decreased the stress-induced secretion of CORT or it inhibited the diurnal rise in CORT since these values were measured in the early dark phase. In efforts to understand this difference, we measured CORT levels in OVX rats exposed to 1, 2, or 3 weeks of daily immobilization stress and found that the CORT response to chronic stress was attenuated by the third week compared to the CORT levels of stressed rats measured in the first week (Cruthirds, Larco, Wu, unpublished data). Interestingly, these results overlap with clinical observations that individuals under severe chronic stress, such as those diagnosed with post-traumatic stress disorder (PTSD), have reduced cortisol levels [55]. Furthermore, a correlation between PTSD and alterations in metabolism has been reported [26], suggesting that our chronic immobilization model may lead to significant alterations in the HPA axis similar to those seen in patients with PTSD.

In the MBH, chronic stress altered leptin signaling. Stressed rats treated with E2 had reduced Ob-Rb levels compared to non-stressed E2-treated rats. As a result, we expected SOCS3 levels to be elevated; however, chronic stress reduced SOCS3 in E2-treated rats in contrast to the increased SOCS3 levels in non-stressed E2-treated rats. SOCS3 is a known negative feedback regulator of leptin and STAT3 signaling [5, 6] leading to a decrease in POMC expression while E2 administration has been shown to up-regulate POMC expression in the ARC [46]. It is possible that in our model of chronic stress, the feedback mechanism governing the expression of SOCS3 was either disrupted or enhanced since we also saw a reversal of the E2-induced increase in Ob-Rb in the non-stressed group compared to stressed E2-treated rats. Furthermore, other players of the HPA axis could be contributing to the stress effects on body weight leading to changes in leptin signaling within the ARC. As already mentioned, we measured serum CORT and we observed that stressed rats had a significantly lower diurnal rise in CORT than non-stressed rats; however, we did not measure CRH, ACTH, or vasopressin, which are involved in the initial steps of HPA activation and may regulate body weight composition. For example, in rodents ICV injection of CRH reduces food intake and is likely mediated by modulating POMC transcription and processing [7, 24, 34]. Furthermore, there is evidence showing leptin increases CRH mRNA expression in the PVN [37] and that treatment with a CRH antagonist blunts effects of leptin on metabolism. These observations indicate that CRH and leptin mediate their actions through a common pathway and may depend on each other to elicit their functions. Further investigation into the CRH and leptin interaction in our chronic stress paradigm is certainly warranted.

In summary, OVX rats treated with E2 had reduced body weight and consumed less food than VEH-treated animals. This effect is in part mediated by E2 enhancing leptin action in the MBH since Ob-Rb levels were elevated. However, chronic E2 treatment may also activate a negative feedback mechanism to modulate the leptin pathway by increasing SOCS3 levels. On the other hand, animals that were exposed to chronic immobilization stress had decreased body weight but only in the absence of E2 treatment. Furthermore, chronic immobilization stress inhibited the E2-induced increase in Ob-Rb and SOCS3 levels in the MBH but these changes did not lead to any differences in body weight composition. Finally, chronically stressed rats had altered HPA axis activity since these rats had diminished levels of CORT at the end of the stress phase but E2 treatment counteracted this effect. The results of the present study suggest chronic stress alters both leptin signaling and HPA activity yet E2 may have multiple mechanisms of maintaining body weight homeostasis.

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